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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/611,949	07/06/2000	David M. Margolis	0184-0001CIP	6524

7590 01/24/2005

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EXAMINER	
GUZO, DAVID	

ART UNIT	PAPER NUMBER
1636	

DATE MAILED: 01/24/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/611,949	MARGOLIS ET AL.	
	Examiner	Art Unit	
	David Guzo	1636	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *30 December 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-23* is/are pending in the application.

4a) Of the above claim(s) *1-10 and 12-23* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *11* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *06 July 2000* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☒ Other: *See Continuation Sheet*.

Continuation of Attachment(s) 6). Other: Notice to Comply with Sequence Rules.

Detailed Action

Applicant's election without traverse of Group XII, claim 11 in the reply filed on 12/30/04 is acknowledged.

Claims 1-10 and 12-23 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 12/30/04.

Sequence Rules

This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 CFR 1.821 through 1.825 for the reason(s) set forth below or on the attached Notice To Comply With Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures. Applicants have not filed a Sequence Listing for this application.

Applicant must comply with the sequence rules, 37 CFR 1.821 - 1.825. Applicant is requested to return a copy of the attached Notice to Comply with the reply. Any reply to this Office Action which does not include complete compliance with the Sequence Rules will be considered non-responsive. The nature of the non-compliance with the Sequence Rules has not precluded an examination of the application on the merits, the results of which are communicated below.

35 USC 112, 1st Paragraph Rejections

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 11 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Applicants claim a method of treating or preventing latent HIV infection in a human subject in need of such treatment or prevention comprising administering to the subject (a) an amount of an inhibitor of an HDAC1-recruiting activity of YYI, said amount being effective to inhibit repression of HIV transcription, and (b) a therapeutically effective amount of one or more anti-viral drugs selected from the group consisting of AZT, 3TC, ddI, ddC, saquinavir, indinavir, ritonavir, nelfinavir, nevirapine and efavirenz.

It appears that applicants' invention involves the following concept. The treatment of HIV using combinations of anti-retroviral drugs (such as AZT, indinavir, ddI, etc.), known as highly active anti-retroviral therapy (HAART), is effective in reducing plasma viremia to undetectable levels. However, replication competent HIV can still be isolated from latently infected $CD4^+$ T cells and suspension of HAART results in viral rebound in patients. Applicants disclosed that HIV gene activation is suppressed, in part, by recruitment of HDAC1 to the viral LTR by the transcription factor YY1 and inhibition of this recruitment can activate HIV gene expression *in vitro*. Applicants'

administration of an agent (in combination with anti-retroviral drugs) that activates HIV gene expression in latently infected cells by interfering with the HDAC1 recruiting activity of YY1 is believed to result in latently infected cells dying from cytopathic effects or immune effector mechanisms while the anti-retroviral agents would prevent new rounds of infection caused by the new virions produced by the activation of the virus in the latently infected cells.

The test of enablement is whether one skilled in the art could make and use the claimed invention from the disclosures in the application coupled with information known in the art without undue experimentation (*United States v. Telectronics, Inc.*, 8 USPQ2d 1217 (Fed. Cir. 1988). Whether undue experimentation is required is not based upon a single factor, but rather is a conclusion reached by weighing many factors. These factors were outlined in *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Inter. 1986) and again in *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988) and include the following:

1) Unpredictability of the art. The art in this area involves the treatment or prevention of latent HIV infection in patients. This art must be considered extremely unpredictable. With regard to the **prevention** of latent HIV infection, the art is not only unpredictable but is also non-existent. With regard to use of agents which are designed to activate HIV expression in latently infected cells in combination with HAART to treat patients, the art is unpredictable. For example, use of IL-2 to activate HIV gene expression in latently infected cells (in combination with antiretroviral agents) to treat latent HIV infection in patients "...has failed to demonstrate a consistent diminution of the pool of latently infected cells or of viral rebound following cessation of therapy..."

(Demonte et al., Biochem. Pharmacology, 2004, Vol. 68, pp. 1231-1238, p. 1236). See also Chun et al., Nature, 1999, Vol. 401, pp. 874-875 for disclosure of failure of attempts to purge latent HIV infection by administering IL-2 and HAART. Indeed, IL-2 appears to activate HIV gene expression by down-modulating the binding activity of YY1 and LSF to the HIV LTR (See Bovolenta et al., J. Immunol., 1999, Vol. 163, pp. 6892-6897). It is noted that an inhibition of binding of YY1 and LSF to the viral LTR would have the same effect on HIV gene activation as inhibiting binding of the HDAC1 to the YY1 since binding of YY1, LSF and HDAC1 to the HIV LTR are apparently required to repress HIV gene expression. Since the art indicates that this approach has failed to treat latent HIV infection, it is unclear how applicants' method would overcome the failures with this approach which are noted in the cited art. It is also noted that recently some researchers have contemplated treatment of latent HIV infections by use of HDAC inhibitors (HDACi) in combination with HAART (See Demonte et al., 2004, cited above); however, this proposal is speculative, remains untried in patients and indeed, Demonte et al. concludes that inclusion of HDACi in treatments merely "deserves further investigations" (p. 1236).

2) State of the art. The art in this area at the time of applicants' invention was nil.

3) Number of working examples. Applicants present no working examples of the claimed invention.

4) Amount of guidance provided by applicants. Applicants present no specific guidance on the dosages of any specific agent which inhibits a HDAC1 recruiting activity of YY1, no specific guidance on the duration of treatment which would be

sufficient to purge the patient of latent HIV, no guidance on how to prevent latent infection of cells by HIV, etc. Since YY1 is an important transcription factor involved in numerous normal biochemical signal pathways, it is important that administration of any inhibitors of YY1 activity not result in deleterious effects in the patient. Applicants present no guidance on how the skilled artisan would choose the appropriate inhibitor of YY1-HDAC1 binding as well as the appropriate dosages and treatment schedules so as avoid negative effects on the patient.

5) Scope of the invention. The scope of the invention involves treatment or prevention of HIV infection (involving any strain of HIV) in human subjects.

6) Nature of the invention. The invention involves the prevention of latent HIV infection, something which has not previously been demonstrated in the art. The invention also involves treatment of latent HIV infection by methodologies (involving disruption of YY1-HDAC1-LSF binding to HIV LTRs) which have not previously been successful.

7) Level of skill in the art. The level of skill in the art is high; however, given the lack of success in treating or preventing latent HIV infection in patients, given the absence of working examples provided by applicants, given the unpredictability of the HIV treatment or prevention art, given the poorly developed state of the art, and given the lack of guidance on specific dosages (and duration of treatment) of inhibitors of YY1 recruitment of HDAC1 to be administered to patients, it must be considered that the skilled artisan would have needed to have practiced essentially trial and error experimentation in order to attempt to practice the claimed invention.

Given the above analysis of the factors which the courts have determined are critical to enablement of a claimed invention, it must be considered that the skilled artisan would have needed to have conducted undue and excessive experimentation in order to practice the claimed invention.

No Claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to David Guzo, Ph.D., whose telephone number is (571) 272-0767. The examiner can normally be reached on Monday-Thursday from 8:00 AM to 5:30 PM. The examiner can also be reached on alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Irem Yucel, Ph.D., can be reached on (571) 272-0781. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

DAVID GUZO
PRIMARY EXAMINER

David Guzo
January 24, 2005

Application No. 09/611949

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 CFR 1.821 - 1.825 for the following reason(s):

☒ 1. This application clearly fails to comply with the requirements of 37 CFR 1.821 - 1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

☒ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 CFR 1.821(c).

☒ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked-up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A substitute computer readable form must be submitted as required by 37 CFR 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 CFR 1.821(e).

☐ 7.

Other: _____

Applicant must provide:

☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing"

☒ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 CFR 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d)

For questions regarding compliance with these requirements, please contact:

For Rules Interpretation, call (703) 308-1123
For CRF submission help, call (703) 308-4212
For PatentIn software help, call (703) 557-0400

Notice of References Cited	Application/Control No. 09/611,949	Applicant(s)/Patent Under Reexamination MARGOLIS ET AL.	
	Examiner David Guzo	Art Unit 1636	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
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	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Demonte et al., Biochemical Pharmacology, 2004, Vol. 68, pp. 1231-1238.
	V	Bovolenta et al., J. Immunol., 1999, Vol. 163, pp. 6892-6897.
	W	Chun et al. Nature, 1999, Vol. 401, pp. 874-875.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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Administration of HDAC inhibitors to reactivate HIV-1 expression in latent cellular reservoirs: implications for the development of therapeutic strategies

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Abstract

The discovery of powerful antiviral compounds in the 90's raised the hope that the human immunodeficiency virus type 1 (HIV-1) might be eradicated. However, if these drugs succeed in decreasing and controlling viral replication, complete eradication of the virus is nowadays impossible. The persistence of virus even after long periods of highly active antiretroviral therapy (HAART) mainly results from the presence of cellular reservoirs that contain transcriptionally competent latent viruses capable of producing infectious particles after cellular activation. These latently infected cells are a permanent source for virus reactivation and lead to a rebound of the viral load after interruption of HAART. Activation of HIV gene expression in these cells combined with an effective HAART has been proposed as an adjuvant therapy that could lead to the elimination of the latently infected cells and then to the eradication of the infection. In this context, we have previously demonstrated that deacetylase inhibitors (HDACi) synergize with TNF-induced NF-κB to activate the HIV-1 promoter. The physiological relevance of the TNF/HDACi synergism was shown on HIV-1 replication in both acutely and latently HIV-infected cell lines. Based on these results, we propose the administration of deacetylase inhibitor(s) together with continuous HAART as a new potential therapeutic perspective to decrease the pool of latent HIV reservoirs by forcing viral expression.

Keywords: HIV-1; Transcription; Latency; Reservoirs; HDCAi; HAART

1. Introduction

1.1. The HIV-1 reservoirs: a major obstacle to the eradication of the virus

The development of highly active antiretroviral therapies (HAART) has dramatically improved the survival and quality of life of HIV-1-infected individuals. Unfortunately, whereas these treatments significantly reduce the levels of viral RNA in plasma and lymphoid tissues, cessation of even prolonged highly suppressive HAART regimens results in viral load rebound to pre-therapy levels, indicating that antiretroviral therapy of this type is unable to completely eliminate HIV-1 [1–3].

This failure has been attributed in part to the presence of a long-lived, stable population of latently infected resting memory $CD4^+$ T cells that are not eliminated by the antiviral treatment [4–6]. Indeed, while many HIV-susceptible cells are fast-turnover cells, this small part of memory T cells are long-lived cells [7,8]. These infected cells can go dormant and stay in tissues for years despite effective HAART, thereby serving as the HIV-1 reservoirs in vivo [6]. These reservoirs have such a slow rate of decay during HAART that their eradication during a human lifespan is unlikely [9,10].

As with all retroviruses, HIV-1 integrates into the genome of the host cell. As a consequence, the activity of the integrated viral genome, or provirus, is greatly influenced by the metabolic and activation state of the host cell. The generation of latency is thought to occur after HIV infection of a transcriptionally active cell, which predominantly

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results in productive infection and cell death. However, if viral transcription decreases before either viral or immunologic cytopathic effects, the virus can become latent (reviewed in [3]). Viral genes expression in these latently infected cells can be reactivated by a wide variety of signals including cytokines such as interleukin-2, tumor necrosis factor alpha/SF2 (referred to as TNF hereafter), macrophage colony-stimulating factor (MCSF), antigens and other T-cell mitogens, glucocorticoid and thyroid hormones, bacterial infections, lipopolysaccharides, and small molecules. Consequently, if HAART is ceased, viremia rapidly re-emerges, regardless of the duration of drug therapy [1,11–13].

It has been proposed that one possible solution to the problem of HIV-1 latency is to purge these reservoirs by deliberately forcing HIV-1 gene expression in these latently infected cells in presence of HAART to prevent spreading of the infection by the newly synthesized viruses [11]. Such treatment could reduce the number of latently infected cells by causing them to be directly killed by the cytopathic action of the virus or to be destroyed by the immune system. The definition of such strategies is clearly dependent on the knowledge of the molecular mechanisms regulating HIV-1 latency and reactivation from latency.

2. Molecular aspects of HIV-1 latency

At the cellular level, two major forms of HIV-1 latency have been described: pre-integration latency and post-integration latency [14]. The first one cannot be taken into account for the formation of the long-term viral reservoirs and will not be further discussed in this review. Among the proposed mechanisms for HIV-1 post-integration latency are: (i) mutations in the Tat-TAR axis [15,16]; (ii) the lack of activation-dependent host transcription factors in resting cells [17–21]; (iii) transcriptional silencing based on chromatin structure at the site of integration [22–25]; (iv) epigenetic modifications (reviewed in [26]).

2.1. Mutations in the Tat-TAR axis

HIV transcription is characterized by two temporally distinct phases. The early phase relies on cellular transcription factors. However, because of a transcriptional elongation defect in the basal HIV-1 promoter, the 5′ long terminal repeat (LTR), most transcripts cannot elongate efficiently and terminate rapidly after initiation. However, a few transcripts elongate throughout the genome, resulting in transcription of the viral transactivator Tat. The late phase of transcription occurs when enough Tat protein has accumulated. Tat binds to TAR, an RNA hairpin loop formed at the 5′ end of all nascent HIV-1 transcripts (reviewed in [27]) (Fig. 1), recruits the pTEFb complex and causes the hyperphosphorylation of the RNA polymerase II, thereby dramatically increasing its ability to elongate. In several latently infected cell lines, which are used as models for studying HIV-1 post-integration latency, the proviruses harbor mutations in their Tat-TAR transcriptional axis [15,16]. Indeed, we have examined the sequence of the Tat protein encoded by proviruses integrated in the latently infected U1 and ACH2 cell lines and the sequence of their respective LTRs. The U1 cell line contains two distinct forms of Tat: one Tat cDNA lacks an ATG initiation codon, while the other contains a mutation at aa 13 (H13L). Both Tat cDNA are defective in terms of transcriptional activation of the HIV-1 LTR [16]. The Tat cDNA amplified from the ACH2 virus is fully functional in terms of transcriptional activation, but the ACH2 LTR contains a point mutation in the Tat responsive element TAR. This latter mutation impairs the Tat-responsiveness of the LTR [15].

Although the significance of such mutants in the generation of HIV-1 latency in infected individuals in vivo has not been established, the studies performed in these latently infected cell lines have led to the concept that transcription inhibition is critical for the establishment and maintenance of HIV-1 latency.

2.2. The NF-κB signaling pathway

The early phase of HIV-1 transcription depends on cellular transcription factors. Therefore, the simplest mechanism for latency involves the absence in resting $CD4^+$ T cells of transcription from the HIV-1 promoter. This could result from the absence in resting cells of host transcription factors that are required for the activation of gene expression from the HIV-1 5′ LTR [17,20,21]. Indeed, the U3 region of the HIV-1 LTR contains binding sites for cellular transcription factors that function as positive regulators of T-cell activation-specific gene expression in normal, uninfected T cells (Fig. 1). The notion that latent infection of T cells might involve the absence of the requisite host transcription factors has been postulated following the demonstration that, in transformed T-cell lines carrying an integrated copy of the HIV-1 genome, up-regulation of HIV-1 gene expression following exposure to TNFα is mediated through nuclear factor-kappa B (NF-κB) [20]. Indeed, the enhancer located in LTR U3 region contains two binding sites for NF-κB, which play a central role in the activation pathway of the HIV-1 provirus (Fig. 1). Various studies have reported that the κB-binding sites as well as the NF-κB proteins are critical for LTR promoter activity and important for optimal HIV-1 replication (reviewed in [28]). In addition, the antigenic stimulation of T cells induces NF-κB activation by stimulation of PKC. Different groups have shown that phorbol ester derivatives [29,30], which induce direct activation of the PKC, potently induce expression of latent viruses in peripheral blood mononuclear cells and in quiescent T cells of infected individuals [31,32]. Induction of other transcription factors, such as AP-1 by the mitogen-activated protein

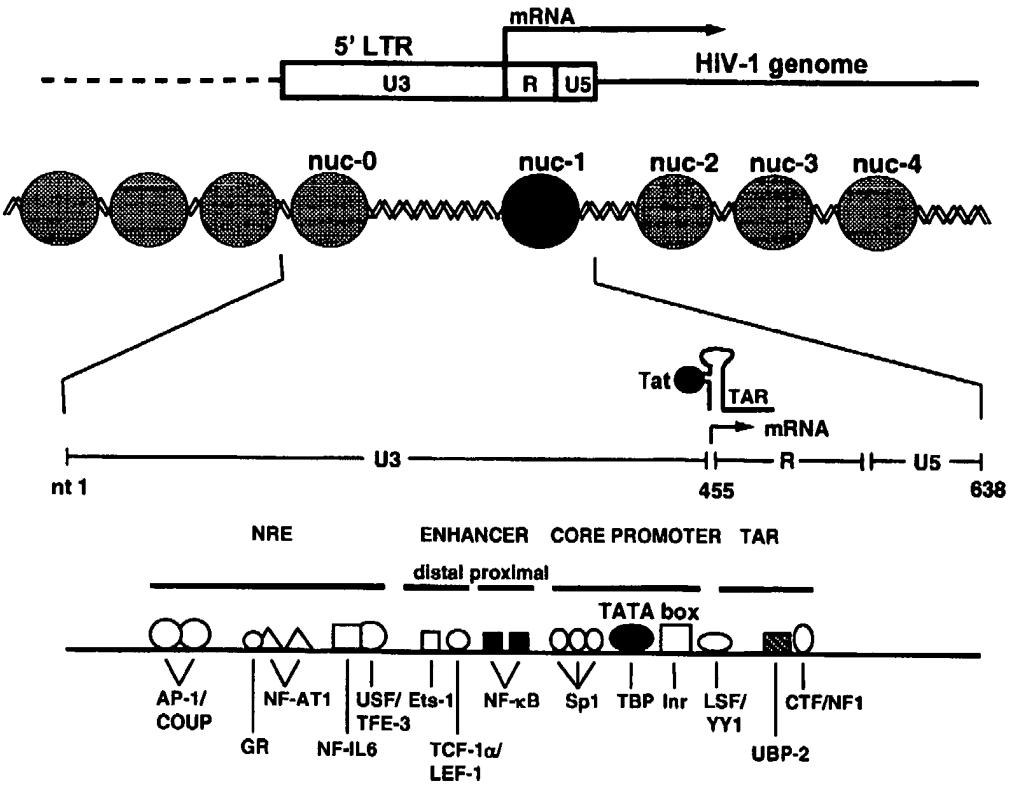

Fig. 1. Transcription elements in the 5'LTR of HIV-1. The U3, R and U5 regions of the LTR and binding sites for several transcription factors, as well as other sequence elements are indicated. Nucleotide (nt) + 1 is the start of U3 in the 5'LTR. The arrow at the U3/R junction denotes the start site of transcription. The region corresponding to the transactivating response (TAR) element is indicated. The mapping of nucleosome positioning in the 5'LTR and leader regions of HIV-1 is also represented. During transcriptional activation, a single nucleosome (nuc-1 in black) located immediately downstream of the transcription start, is specifically remodeled. This change is the only one observed in the complete genome of HIV-1 upon transcriptional activation.

kinase pathway and Sp1, also activate HIV LTR activity. However, their action is largely enhanced via interaction with NF-κB [33,34]. Like NF-κB, activation of the transcription factor nuclear factor of activated T cells (NF-AT) is induced early after T-cell stimulation and is critical to T-cell activation and proliferation [35].

2.3. The chromatin structure

Following infection, the HIV-1 proviral DNA is integrated into the host genome. Here, chromatin conformation seems to repress transcription from the integrated LTR promoter (reviewed in [36,37]). We have previously shown that, independently from the integration site, nucleosomes in the 5′ LTR are precisely positioned [25]. In the transcriptionally silent provirus, these nucleosomes define two large nucleosome-free regions. The first region corresponds to the enhancer/promoter area and the second region spans the primer-binding site region immediately downstream of the 5'LTR. These two open regions of chromatin are separated by a single nucleosome called nuc-1, that is specifically and rapidly disrupted during transcriptional activation (Fig. 1). The position of nuc-1 in the close proximity of the transcription start site and its disruption/remodeling during transcriptional activation led us to postulate that chromatin plays a crucial role in the repression of HIV-1 transcription during latency and that nuc-1 disruption is necessary for transcriptional activation [24,25]. In addition, studies from David Margolis group have demonstrated that the host factors YY1 and LSF cooperatively recruit histone deacetylase 1 (HDAC1) to the HIV-1 LTR and inhibit transcription by maintaining nuc-1 in an hypoacetylated state [38,39]. In agreement with their data, pyrole-imidazole polyamides, which block the binding of the LSF–YY1 complex to the LTR and consequently the recruitment of HDAC-1 close to nuc-1, have been shown to induce reactivation of HIV-1 expression [38,39]. These experiments highlight the repressive role played by the nucleosome nuc-1 in the generation of post-integration latency. However, although the chromatin structure adopted by the integrated HIV-1 provirus is independent of the integration site, it has been demonstrated that the location of the provirus in hetero- or euchromatin locus could be an additional element to consider in the generation of post-integration latency [22,23].

2.4. Acetylation of histones and non-histone proteins

There is now strong evidence that both transcriptional activation and silencing are mediated at least in part

through the recruitment of enzymes that control protein acetylation. Acetylation of specific lysine residues within nucleosomal histones is generally linked to chromatin disruption and transcriptional activation of genes. Consistent with their role in altering chromatin structure, many transcriptional co-activators possess intrinsic histone acetyltransferase (HAT) activity that is critical for their function (reviewed in [40]). Similarly, co-repressor complexes include proteins that have histone deacetylase (HDAC) activity (reviewed in [41]). Importantly, reversible acetylation is also a critical post-translational modification of non-histone proteins, including general and specific transcription factors, co-activators, non-histone structural chromosomal proteins, and nuclear import factors. In the case of HIV-1, there is ample evidence that viral transcription is regulated by protein acetylation. Indeed, we and others have demonstrated the transcriptional activation of the HIV-1 promoter in response to deacetylase inhibitors (HDACi) (such as trichostatin A (TSA), trapoxin (TPX), valproic acid (VPA) and sodium butyrate (NaBut)). This occurs in ex vivo transiently or stably transfected HIV-1 LTR promoter reporter constructs [42–45], in latently HIV-1-infected cell lines [44–47], on in vitro chromatin-reconstituted HIV-1 templates [48,49], as well as in the context of a de novo infection [45]. HIV-1 transcriptional activation following treatment with HDACi is associated with nuc-1 remodeling (reviewed in [36]). Moreover, it has been demonstrated that acetylation of the HIV-1 transactivator Tat by p300, by P/CAF, and by human GCN5 is important for its transcriptional activity (review in [26,50–52]).

HDACi, such as TSA, strongly induce HIV-1 transcription, although only a small subset (<2%) of cellular genes have their expression modified by TSA [53]. The ability of the HIV-1 promoter to respond specifically to TSA might reflect a complex regulatory link between deacetylation and transcriptional repression of the LTR and highlights the importance of protein acetylation in the regulation of HIV-1 expression and especially in its reactivation from latency.

2.5. A regulatory link between NF-κB signaling pathway and protein acetylation

The transcription factor NF-κB and the regulation of protein acetylation both play a central role in the HIV-1 activation pathway. To better understand the molecular mechanisms regulating HIV-1 reactivation from latency, we have recently extended our studies on the TSA inducibility of the viral promoter (LTR) and focused on the functional role of the κB sites in this TSA response. We have demonstrated that HDACi (such as TSA and NaBut) synergize with TNF-induced NF-κB to activate transcription from the HIV-1 LTR [45]. This synergism requires intact κB sites and was observed with LTRs from subtypes A to G of the HIV-1 group M (major) [45]. Importantly, the physiological relevance of the TNF/TSA(NaBut) synergism was shown both on HIV-1 reactivation in a model cell line for post-integration latency and on HIV-1 replication in the context of a de novo viral infection [45].

Therefore, our results open new therapeutic strategies aimed at forcing viral expression and at contributing, in the presence of an efficient HAART, to a reduction of the pool of latently HIV-infected cellular reservoirs (see Section 3 here below).

We have unraveled the molecular mechanisms underlying the TNF/TSA synergism. In mammalian cells, there are five known members of the NF-κB/Rel family: p65 (RelA), c-Rel, RelB, p50, and p52. The most widely studied and most abundant form of NF-κB is a heterodimer of p50 and p65. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through interaction with its inhibitor IκBα, but NF-κB can be induced transiently by various stimuli (including inflammatory cytokines (TNFα, IL-1), bacterial lipopolysaccharides, viral proteins, mitogens, UV light) (Fig. 2).

Mechanistically, we have demonstrated that TSA and NaBut prolonge TNF-induced NF-κB binding to DNA and the intranuclear presence of p65 [54]. We have shown that the p65 subunit of NF-κB is acetylated in vivo. However, this acetylation is weak, suggesting that other mechanisms could be implicated in the potentiated binding and transactivation activities of NF-κB after TNF + TSA versus TNF treatment. Western blot and immunofluorescence confocal microscopy experiments have revealed a delay in the cytoplasmic reappearance of the IκBα inhibitor, which correlated temporally with the prolonged intranuclear binding and presence of NF-κB [54]. This delay was due neither to a defect in IκBα mRNA production, nor to a nuclear retention of IκBα, but rather to a persistent proteosome-mediated degradation of IκBα. We have demonstrated that TSA prolongs the activity of the IkB kinase (IKK) complex [54]. This prolongation of IKK activity could explain, at least partially, the delayed IκBα cytoplasmic reappearance observed in presence of TNF + TSA and the TNF/TSA synergism observed on the HIV-1 promoter [54] (Fig. 2).

3. HDACi: a new hope for HIV-1 eradication?

We have demonstrated a synergistic effect of TNF and HDACi on reactivation of HIV-1 expression in the latently infected U1 cell line (see Section 2.5). In addition, our preliminary experiments performed on CD8-depleted PBMCs isolated from aviremic patients suggest that HDACi are able to reactivate HIV-1 expression in latently infected cells (unpublished results). It is important to note that an array of cytokines, including the proinflammatory cytokines TNF and interleukin-1 (inducers of NF-κB), are already copiously expressed in the microenvironment of the lymphoid tissues, which harbor latent viral reservoirs [55]. Therefore, our results suggest that the use of

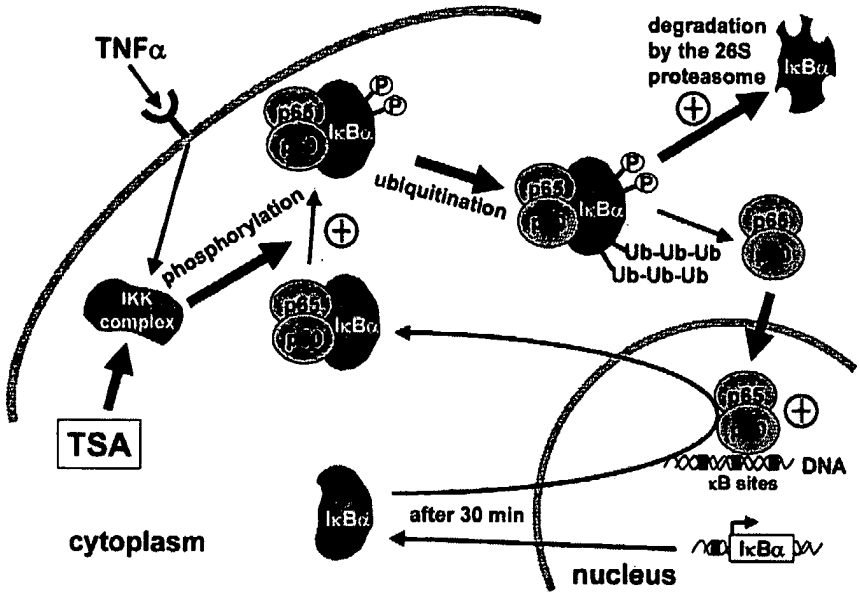

Fig. 2. The NF-kB signaling pathway and the effect of TSA. In unstimulated cells, NF-κB (represented by the protein dimer p50/p65) is sequestered in the cytoplasm in an inactive form through interaction with inhibitory IκB proteins including IκBα, IκBβ and IκBε. Upon activation of NF-κB by various stimuli (including inflammatory cytokines (TNFα, IL-1), bacterial lipopolysaccharides, viral proteins, mitogens, UV light), IκBs are rapidly phosphorylated by a macromolecular IκB kinase (IKK) complex, ubiquitinated and degraded by the 26S proteasome. The released NF-κB then translocates to the nucleus, where it can activate transcription from a wide variety of promoters, including that of its own inhibitor IκBα. The newly synthesized IκBα enters the nucleus, enhances NF-κB removal from DNA, and takes it back to the cytoplasm, thus restoring the inducible cytoplasmic pool of NF-κB. Thus, the de novo expression of IκBα proteins, which display nucleocytoplasmic shuttling properties, participates in a negative feedback system ensuring a transient NF-κB transcriptional response. Mechanistically, we have demonstrated that TSA prolongs the activity of the IKK complex (indicated by a + sign) and thus prolongs the phosphorylation of IκBα. The phosphorylation of IκBα by IKK is critical for its proteasome-mediated degradation. Therefore, the prolongation of IKK activity could explain the persistent degradation of neosynthesized IkBα observed in presence of TNF + TSA (indicated by a + sign) and the resulting prolonged presence of NF-κB in the nucleus (indicated by a + sign).

deacetylases inhibitors in the treatment of HIV infection may represent a valuable approach for purging the latently infected reservoirs in HAART-treated individuals. These HDACi would synergize with the cytokines already present at increased level in the serum of the HIV-infected individuals.

HDACi present several advantages in the context of an anti-HIV-1 adjuvant therapy. First, an ideal adjuvant agent would induce expression of HIV-1 without inducing global T-cell activation to prevent the generation of new target cells for the neo-synthesized virus. Indeed, HDACi do not induce proliferation or activation of T cells [56–58]. Recent data even suggest that HDACi inhibit CD4 T-cell proliferation in a dose-dependent manner [59]. Second, although infected resting $CD4^+$ T cells represent the major long-term HIV-1 reservoirs, other cell types (macrophages, dentritic cells and other non-T cells) clearly contribute to the persistence of HIV-1 during HAART [60,61]. As viral suppression in T cells becomes today increasingly effective, these alternative reservoirs may take on even greater relative importance as sites for viral persistence and as targets for purging. HDACi have been shown to act in a broad spectrum of cell lines and therefore, in contrast to agents that specifically induce T cells, they could target the different cellular HIV-1 reservoirs. Third, this class of agents is safely administrated for several years for other diseases, including beta-chain hemoglobinopathies (such as beta-thalassemia and sickle cell anemia) [62,63], epilepsy and bipolar disorders [64–66]. Moreover, HDACi are potent inducers of apoptosis and growth inhibition in transformed cells originating from lymphoid cells [56–58]. As HDACi are relatively non-toxic to normal cells, they are now considered as good candidates for novel cancer therapy. Among the different agents that have been identified as having HDACi activity (including TSA, TPX, oxamflatin, apicidin, phenylbutyrate, suberoylanilide hydroxamic acid (SAHA), pyroxamide, and FR901228), at least the latter four are currently used in clinical trials for evaluation of their anticancer efficacy [67].

The fourth element in favour of the use of HDACi in antiHIV treatment is the ability of these drugs to induce the transcriptional activation of several HIV-subtypes LTRs. Indeed, an increasing number of non-B HIV-1 subtype infections are currently diagnosed. We have shown that, in addition to the prototypical subtype B LTR, the LTRs from subtypes A to G of the HIV-1 major group M were also activated synergistically by TSA and TNF, and that the amplitude of the synergism correlates with the number of κB sites in the respective LTRs, which varies from one (subtype E) to three (subtype C) [45]. These data suggest that HDACi could be used to induce HIV-1 expression in a subtype non-specific manner.

A fifth important element is the fact that HDACi are already safely administrated to HIV-1 patients for years. Indeed, it is well documented that patients with HIV-1 are at an increased risk for the development of multiple neurologic manifestations including seizures [68]. Therefore, HIV-positive patients typically receive long-term anticonvulsant therapy following an initial episode of seizure activity [69]. Historically, these patients have been treated with many anticonvulsant agents including valproic acid which possess, in addition to its neurologic properties, HDACi activities. Supporting our hypothesis based on the ability of HDACi to induce HIV-1 replication in vivo, an increase in viral load of some HIV-positive patients receiving both HAART and valproic acid has been observed [70]. However, this phenomenon has not been observed in all patients receiving this combinatory therapy [69], suggesting that in some patients the increase in the viral load can be controlled by the antiretroviral treatment.

4. Discussion

HIV-1 latency represents a major problem in the eradication of HIV-1 in infected individuals treated with HAART. Indeed, even though the antiretroviral drugs protect uninfected cells from HIV infection, the HIV-infected cells are not affected by this therapy. In the absence of virus gene expression, latently infected cells differ from their uninfected counterparts by only the presence of 10 kb of viral DNA integrated into the host cell genome. These cells are not eliminated by the immune system or by viral-induced death. These reservoirs are thought to consist mainly of latently infected resting memory $CD4^+$ T cells and show striking stability, with a $t_{1/2}$ of 44 months. At this rate of decay, eradication of a reservoir consisting of only 10^6 memory $CD4^+$ T cells would take 73 years [10]. One possible solution to the problem of HIV-1 latency is to deliberately administrate agents that activate viral gene expression in the presence of HAART to prevent the spreading of the infection by the newly synthesized viruses [11]. The ideal HIV-1 inducing agent would be potent, orally available, non-toxic, active in a wide variety of latently infected cell types, and capable of penetrating anatomic sanctuaries such as the central nervous system. HDACi present such properties: (i) they are potent inducers of the HIV-1 expression; (ii) they can induce viral replication in different cell types and activate transcription of different HIV-1 subtype LTRs; (iii) some HDACi are already used in therapy against various diseases and several new molecules are under clinical trials; (iv) the HDACi valproic acid has already be given to HIV-positive patients for the treatment of neurological pathologies. These latter studies have demonstrated the ability of this molecule to cross the blood/brain barrier and the possibility to use HDACi as adjuvants to HAART.

Clinical efforts to reduce the latent HIV-1 reservoirs have so far been largely unsuccessful. Although treatment with interleukin-2 improves $CD4^+$ T-cell counts and reduces the HIV-1 reservoirs in some patients, systematic clinical studies have failed to demonstrate a consistent diminution of the pool of latently infected cells or of viral rebound following cessation of therapy [71,72]. Attempts to improve the ability of the endogenous immune system to combat HIV-1 by structured therapy interruptions (which are defined as a precise schedule according to which patients are put on and off therapy over a defined period of time) have also failed to reduce viral reservoirs and may actually promote the emergence of drug- and CTL-resistant viruses [73]. Cytoreductive therapy with cyclophosphamide in combination with HAART has also not reduced the cellular reservoirs of the virus [74]. Given these circumstances, together with the emerging problems of drug resistance and toxicity due to HAART, the possibility of treating persistent HIV-1 infection by inductive therapy with HDACi deserves further investigations.

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In Vivo Administration of Recombinant IL-2 to Individuals Infected by HIV Down-Modulates the Binding and Expression of the Transcription Factors Ying-Yang-1 and Leader Binding Protein-1/Late Simian Virus 40 Factor[1]

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Leader binding protein-1 (LBP-1)/late SV40 factor (LSF) and ying yang-1 (YY1) transcription factors are involved in the regulation of HIV expression. In particular, YY1 and LBP-1 have been shown to cooperate in repressing HIV-1-long terminal repeat reporter gene expression by in vitro cotransfection experiments. However, no information is available on the levels of expression and activation of these transcription factors in PBMC of HIV-infected individuals. Therefore, we have evaluated the expression and DNA binding activity of YY1 and LBP-1 (LSF) in PBMC of HIV-infected individuals before, during, and after administration of IL-2 in association with antiretroviral therapy (ART), a regimen under consideration for broad clinical use in this disease based on its ability to stably raise the absolute number of circulating $CD4^+$ T lymphocytes. Both YY1- and LBP-1 (LSF)-DNA binding were profoundly down-modulated during administration of IL-2/ART, and a proteolytic activity probably responsible for the reduced expression of the two cellular transcription factors was found activated in PBMC of individuals receiving the immunotherapeutic regimen. This study is the first evidence of modulation of cellular transcription factors following IL-2/ART administration and provides a potential correlate of the transient raises in plasma viremia early reported in patients receiving IL-2 in the absence of ART, thus underscoring the importance of always administering this cytokine to HIV-infected individuals together with potent antiretrovirals. *The Journal of Immunology,* **1999, 163: 6892–6897.**

Transcription of HIV is governed by the coordinated and complex action of both viral and cellular transcription factors acting via binding to its long terminal repeats (LTR)[3] (1–4). Both activators, such as NF-κB, Sp1, NF-ATc, and cAMP response element binding protein, and repressors, such as leader binding protein-1 (LBP-1) (5, 6) (also known as late SV40 factor (LSF)) and ying yang-1 (YY1), have been described to modulate HIV-LTR-driven transcription, respectively (2, 3). In contrast to an abundant literature concerning induction and modulation of positive transcription factors, mostly focusing on activation of NF-κB (7, 8), little is known about the expression and modulation of negative transcription factors. In this regard, LBP-1 is a nuclear factor that was initially identified as a 63-kDa polypeptide called LSF (9, 10), because of its ability to stimulate transcription from the SV40 major late promoter (10), which is rapidly phosphorylated upon mitogenic stimulation of human T cells (11). Sequence analysis revealed that LSF is identical with LBP-1c, which is one of the four proteins (LBP-1a, -b, -c, and -d) encoded by the cDNAs generated by alternative splicing of two related genes (one gene encoding the LBP-1a and -b and the other encoding the LBP-1d and -c products, respectively) (12). LBP-1 binds to the −16 to +27 HIV-LTR sequence, including the initiation site and the TATA box (13). Mutations of distinct sites in the −2 to +21 region of the HIV-LTR, encompassing the LBP-1 (LSF)-DNA binding element, did not affect either the basal or Tat-induced HIV-LTR-driven transcription in transient transfection experiments (14). However, it was later demonstrated that LBP-1 (LSF) indeed repressed HIV expression by in vitro transcriptional analysis (12). In the same study, however, immunodepletion of LBP-1 (LSF) failed to show any effect, suggesting that another factor(s) could bind to the same region of the viral LTR (12).

YY1 is a multifunctional, nuclear zinc finger, 68-kDa protein (15–18) that can activate, repress, or initiate the transcription of several cellular genes, including IFN-γ, β-casein, c-*fos*, and c-*myc*, as well as the promoters of several viruses, including CMV, Moloney murine leukemia virus, human papilloma virus, and HIV-1 (19, 20). Of interest, YY1 was shown to bind the same region (−17 to +27) in the HIV-LTR recognized by LBP-1 (21). Recently, a heterodimeric complex composed of the two transcription factors, YY1 and LBP-1 (LSF), was demonstrated to bind to the −17 to +27 HIV-1-LTR element (22). In addition, YY1 and LBP-1 were shown to cooperate in repressing HIV-LTR expression in in vitro cotransfection experiments (22).

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[3] Abbreviations used in this paper: LTR, long terminal repeat; ART, antiretroviral therapy; YY1, ying yang-1; LBP-1, leader binding protein-1; LSF, late SV40 factor; PI, protease inhibitor; RTI, reverse transcriptase inhibitor; MIU, millions of international units; WCE, whole cell extract region; UCR, upstream conserved

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Table I. *Percentage of PBMC subpopulations in HIV-1-infected individuals receiving IL-2*

IL-2[a]	CD3$^+$	CD4$^+$	CD8$^+$	CD14$^+$	CD16$^+$	CD19$^+$
Patient 1						
Screening	67	32	33	14	9	17
1_I*	72	32	37	11	11	12
3_I	70	17	51	16	8	16
5_I	73	26	44	15	11	14
14_I	71	35	30	8	10	12
1_{VI}	78	58	18	7	10	8
3_{VI}	65	36	28	9	16	14
5_{VI}	64	42	19	18	19	7
Patient 2						
Screening	84	31	52	9	2	12
1_I	86	27	57	10	3	10
3_I	85	20	60	14	3	8
5_I	83	25	55	8	5	9
14_I	87	32	55	7	3	9
1_{VI}	89	48	40	5	3	7
3_{VI}	86	34	42	4	4	5
5_{VI}	82	38	42	10	7	2

[a] The arabic number indicates the day within the cycle of IL-2 administration; the cycle numbers are indicated as subscripts.

In contrast to the solid in vitro evidence of the involvement of YY1 and LBP-1 (LSF) in the modulation of HIV-LTR, no information on the state of expression and DNA binding of these transcriptional regulators in cells of HIV-infected individuals has been reported to date. Because LBP-1 (LSF)-DNA binding activity in T lymphocytes can be enhanced by mitogenic stimulation in vitro (11), we investigated whether in vivo IL-2 administration could somehow affect both LBP-1 (LSF) and YY1-DNA expression and binding activities in PBMC. In this regard, IL-2 is a cytokine responsible for the activation, proliferation, and differentiation of T lymphocytes and other immune cells (23) that has been previously shown to increase the absolute number of circulating CD4$^+$ T cells in HIV$^+$ individuals in a stable manner to normal or near normal levels (24–27). However, transient peaks of HIV-1 replication after infusion of IL-2 in the absence of antivirals have been clearly documented (24), and since then, administration of the cytokine has been always associated with potent antiretroviral therapy (ART) (28). In the present study we investigated the state of activation of YY1 and LBP-1 (LSF) before, during, and after administration of IL-2/ART to HIV-infected individuals in vivo. We have observed that YY1- and LBP-1 (LSF)-DNA bindings are profoundly down-modulated by IL-2 administration; we also found that a proteolytic activity is induced in PBMC by administration of this cytokine in vivo, and it is probably responsible for the diminished expression of the two cellular transcription factors.

Materials and Methods

Patients

Six HIV-infected individuals (three women and three men; age, 21–60 years; average, 40 years) were chosen among patients enrolled in a randomized phase II study of administration of rIL-2 (Proleukin, Chiron, Emeryville, CA) after obtaining signed informed consent. All HIV-seropositive individuals had CD4$^+$ T cell count between 200–500 cells/mm^3 and were antiviral-experienced at study entry. After enrollment, they received a mixture of antivirals, consisting of a combination of a protease inhibitor (PI) (Saquinavir) plus two reverse transcriptase inhibitors (RTI) together with IL-2. All tested individuals but one received 3 million international units (MIU) of IL-2 twice daily s.c. for 5 days of a 5-wk treatment cycle for a total of 12 cycles; one individual received ART plus 15 MIU by continuous infusion for 5 days for two cycles with an 8-wk interval, followed by four cycles of 7.5 MIU twice daily for 5 days. The cumulative amount of IL-2 administered over 12 mo was equal for all patients. These individuals belonged to an open-label trial designed as a four-arm study enrolling 15 individuals/arm, in which the control group received antiretrovirals (two RTI and one PI) only. The results of the trial, fully supporting the superiority of IL-2-containing regimens vs ART alone in terms of reconstitution of physiologic levels of circulating CD4$^+$ T lymphocytes (Table I), are being reported separately (our manuscript in preparation). PBMC were isolated from either peripheral venous blood of HIV-infected individuals or buffy coats of healthy normal donors by Ficoll-Hypaque gradient sedimentation (Pharmacia Biotech, Uppsala, Sweden), as previously described (29). Aliquots of 1×10^6 cells were washed twice with RPMI 1640 (Life Technologies-BRL, Grand Island, NY) and spun at 13,000 $\times$ *g* for 2–3 min, and the pellets were stored at −80°C for further analyses.

Antibodies

Affinity-purified rabbit polyclonal Ab raised against a C-terminal epitope of human YY1 (sc-281) was purchased from Santa Cruz Biotechnology (S. Cruz, CA); rabbit polyclonal Ab against human actin (A2066) was purchased from Sigma (St. Louis, MO).

Whole-cell extracts (WCE) and EMSA

WCE were prepared by repeated freeze-thaw cycles, as previously described (30). Briefly, the cell pellets were resuspended in high salt buffer C containing 0.1% Nonidet P-40, a mixture of protease inhibitors that included leupeptin (10 μg/ml), pepstatin A (10 μg/ml), aprotinin (33 μg/ml), E-64 (10 μg/ml), Pefabloc 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF; 1 mM), di-isopropylfluorophosphate (3 mM), and the phosphatase inhibitors sodium vanadate (1 mM), and sodium fluoride (50 mM). Following three cycles of freezing and thawing, cellular debris were pelleted by centrifugation at 1200 $\times$ *g* for 15 min at 4°C. The resulting supernatants (WCE) were stored at −80°C. The protein concentration was measured using a kit based on the Bradford method (Bio-Rad, Hercules, CA). EMSA was performed as previously described (30), with minor modifications, in that binding buffers appropriate for each specific oligonucleotide were used. In particular, for the LSF-280 probe, corresponding to the LSF binding site (+260 to +301) within the SV40 major late promoter (5′-ACA CAC ATT CCA CAG CTG GTT CTT TCC GCC TCA GAA GGT ACC TAA C-3′) (10), a binding buffer containing 20% glycerol, 1.2% Nonidet P-40, 127 mM KCl, 8 mM Tris-HCl (pH 7.9), 20 mM HEPES (pH 7.9), 0.18 mM EDTA, and 0.86 mM DTT was used (11). For the UCR probe, corresponding to the YY1 binding site within the Moloney murine leukemia virus promoter (5′-CTG CAG TAA CGC CAT TTT GCA AGG CAT GAA-3′) (18), a binding buffer containing 10% glycerol, 10 mM Tris-HCl (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT was adopted. For the prolactin-responsive element probe, located within the β-casein promoter (TAG ATT TCT AGG AAT TCG) (31), a binding buffer containing 10 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol was used. The DNA-protein complexes were resolved on PAGE as previously described (30).

Immunoblot analyses

Immunoblot analyses were performed as previously described (30). Anti-YY1 and anti-actin Abs were diluted 1/2000 and 1/500, respectively, following the manufacturer's instructions. HRP-conjugated anti-mouse or anti-rabbit secondary Abs were diluted 1/5,000 and 1/15,000, respectively. The signal was revealed by the enhanced chemiluminescence system (ECL, Amersham, Aylesbury, U.K.) following the manufacturer's instructions.

Results

In vivo IL-2 administration to HIV$^+$ individuals suppresses YY1 and LBP-1 (LSF) binding to target DNA sequences

Binding of YY1/LBP-1 (LSF) complex to the HIV-1-LTR element has been previously demonstrated using WCE obtained from uninfected cells (22). Therefore, we have incubated WCE obtained from PBMC of HIV$^+$ individuals isolated both before and during in vivo IL-2 administration with either the canonical YY1 DNA binding element, UCR, present in the Moloney murine leukemia virus promoter (18), or the canonical LBP-1 (LSF)-DNA binding element, LSF-280, of the SV40 major late promoter. Strong binding of YY1 to the UCR element was demonstrated before the initiation of IL-2 therapy (time zero; Fig. 1*A*), whereas IL-2 administration was associated with a profound down-modulation of YY1/UCR binding activity, which, however, returned to a detectable level at suspension of cytokine administration (intercycle; Fig. 1*A*). Of interest, the transient disappearance of YY1 binding to the

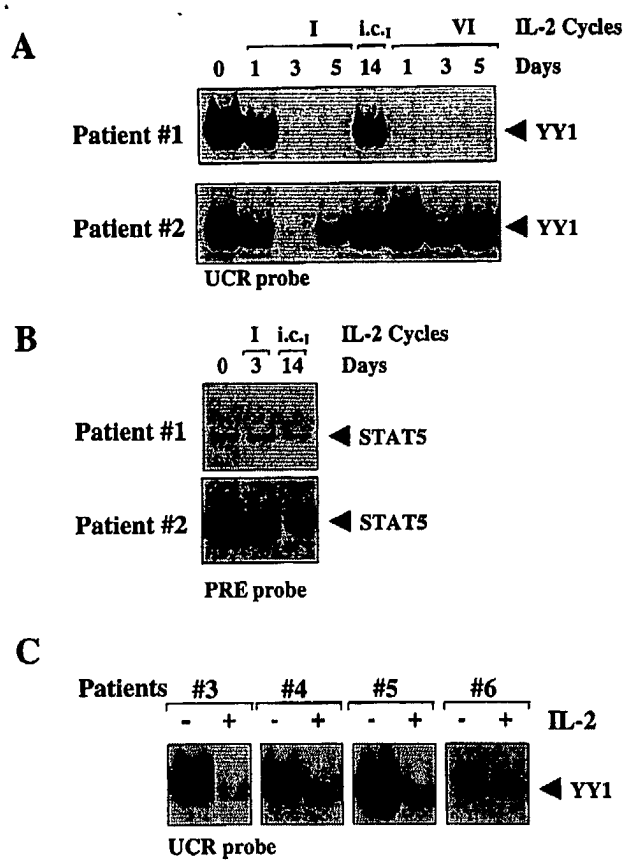

FIGURE 1. *A*, Down-modulation of YY1 binding to UCR probe by IL-2 administration in vivo. The EMSA was performed using 8 μg of WCE obtained from PBMC isolated at different time points during two cycles of IL-2 administration from two HIV-seropositive patients who either highly (donor 1) or poorly (donor 2) responded to IL-2 treatment in terms of $CD4^+$ cells recovery. Day 14 was the day analyzed in between two cycles (IC, intercycle). *B*, STAT5 DNA binding activity is not down-modulated by IL-2 administration in vivo. EMSA using 8 μg of WCE obtained from PBMC isolated before and on day 3 of the two cycles of IL-2 administration from patients 1 and 2 and the prolactin-responsive element probe that specifically binds STAT5. C, EMSA using 8 μg of WCE obtained from PBMC isolated at time zero (−) and on day 3 of cycle 1 (+) of IL-2 administration. All individuals responded to IL-2/ART in terms of increases in $CD4^+$ T cell counts of 7-, 1.5-, 1.7-, and 4.7-fold vs baseline during cycle 5 for patients 3, 4, 5, and 6, respectively.

UCR during IL-2 administration was much more evident in an individual (patient 1) who showed a greater increase in circulating $CD4^+$ T cells after five cycles (1728 vs 477 cells/μl at baseline; with an absolute increase of 3.6-fold, respectively) in response to IL-2 than in a second individual (patient 2), who only moderately responded after 10 cycles (with 731 vs 320 cells/μl at baseline; with an absolute increase of 2.2-fold, respectively). Of note, the lack of YY1 binding was already present on day 1 of the sixth cycle of IL-2 administration in patient 1, whereas no substantial modulation of YY1 binding was simultaneously observed in patient 2 (Fig. 1A). To exclude nonspecific degradation due to improper preparation of WCE, selected time points have been tested for DNA binding activity of the transcription factor STAT5, which we have recently demonstrated to be constitutively activated in the majority of HIV^+ individuals (32). No significant difference was observed before or after IL-2 administration in both patients (Fig. 1*B*). The analysis of four other individuals revealed that three of them responded to IL-2 therapy similarly to patient 1, whereas

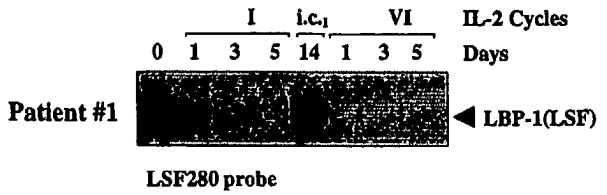

FIGURE 2. Down-modulation of LSF/LBP-1 binding to LSF-280 probe by IL-2 administration in vivo. The EMSA was performed using 8 μg of WCE obtained from PBMC isolated at different time points during two cycles of IL-2 administration from donor 1, who highly responded to IL-2 administration.

weak binding activity and no substantial modulation by IL-2 were observed in one individual (patient 6), as shown in Fig. 1*C*. These results demonstrate that IL-2 administration either directly or indirectly causes a reversible disappearance of YY1-DNA binding. We next examined whether the LBP-1 (LSF)-DNA binding activity was also affected by IL-2 administration by using the radiolabeled LSF280 oligonucleotide and WCE of patient 1. As shown in Fig. 2, down-regulation of LBP-1 (LSF)-DNA binding activity mirrored that of YY1-UCR binding both during cycle 1 and after cycle 6.

To analyze whether cell stimulation by IL-2 in vitro could induce similar patterns of YY1- and LBP-1 (LSF)-DNA binding down-modulation, EMSA analyses were performed using WCE obtained from PBMC of either HIV-seronegative healthy donors or HIV-infected individuals. The cells were either left unstimulated or stimulated for 1, 3, and 5 days with IL-2 (20 U/ml). DNA binding of both transcription factors was readily demonstrated after 24 h in culture and was not down-modulated by the cytokine treatment in vitro (data not shown). These findings indicate that a factor(s) induced in vivo by IL-2 administration is probably responsible for the observed down-modulation of YY1 and LBP-1 (LSF) binding to target DNA.

IL-2 induces a proteolytic activity responsible for YY1-DNA binding down-modulation

We investigated whether the disappearance of YY1 DNA binding activity in WCE of IL-2-treated individuals was caused by a proteolytic activity. To test this hypothesis, WCE from a normal healthy donor seronegative for HIV was mixed with UCR probe alone or in the presence of increasing amounts of WCE from patient 1 obtained on day 5/cycle 1 either at room temperature or at 37°C for 30 min. Normal WCE showed a band in EMSA corresponding to YY1 binding at both room temperature and 37°C (Fig. 3*A*, *lanes 1* and *6*, respectively), whereas WCE from the IL-2-treated individual showed a band that migrated much faster than that observed with the WCE of a normal donor (Fig. 3*A*, *lane 1* vs *lane 11*, respectively), probably corresponding to a truncated form of YY1 (YY1Δ). At room temperature, mixing different amounts of the WCE of the HIV^+ and HIV^- individuals resulted in a decrease in the intensity of the YY1 band at all concentrations of IL-2-treated WCE added, and, in parallel, in the appearance of a band migrating at the same level of the IL-2-treated patient's WCE (Fig. 3*A*, *lanes 2–5*). At 37°C, the disappearance of the upper band corresponding to full-length YY1 was almost complete at the highest concentrations of IL-2-treated WCE (Fig. 3*A*), as demonstrated by quantitative analysis performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA; Fig. 3*B*).

IL-2-induced proteolytic cleavage of YY1 is specific

To assure that the proteolytic cleavage of YY1 indeed occurred in individuals receiving IL-2, Western blot analyses of WCE from

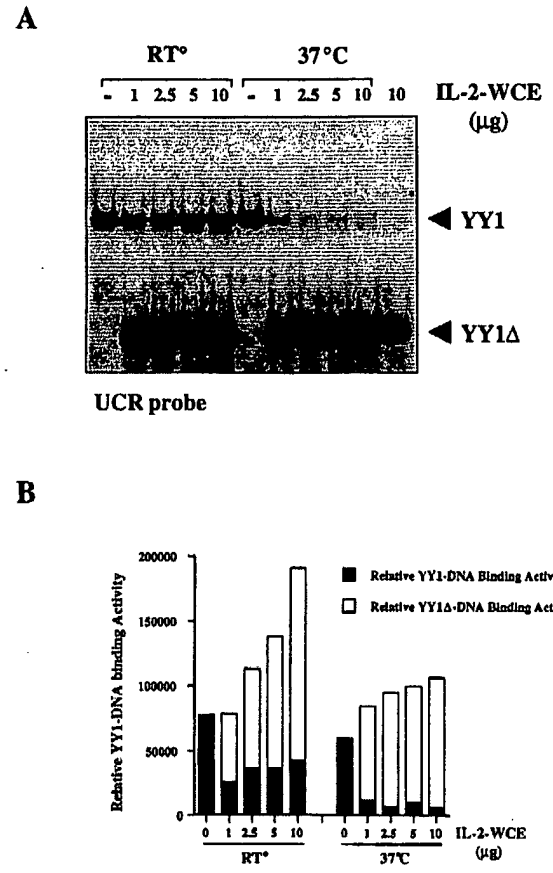

FIGURE 3. Inhibition of YY1 DNA binding activity of a normal healthy donor by the addition of IL-2-treated WCE of a seropositive patient to the binding reaction. *A*, EMSA using 2.5 μg of WCE from a normal healthy donor either alone (−) or in combination with increasing amount of WCE from donor 1 on day 5 of cycle 1. In the *last lane*, 10 μg of WCE from donor 1 on day 5 of, cycle 1 was incubated at room temperature with the probe. *B*, Relative estimates of YY1-DNA binding complex. Quantitation of the relative DNA binding activity of EMSA in *A* was performed by PhosphorImager analysis. Black bars represent the DNA binding activity of full-length YY1 (slower migrating band), whereas white bars indicate that of the truncated YY1 protein (faster migrating band, YY1Δ).

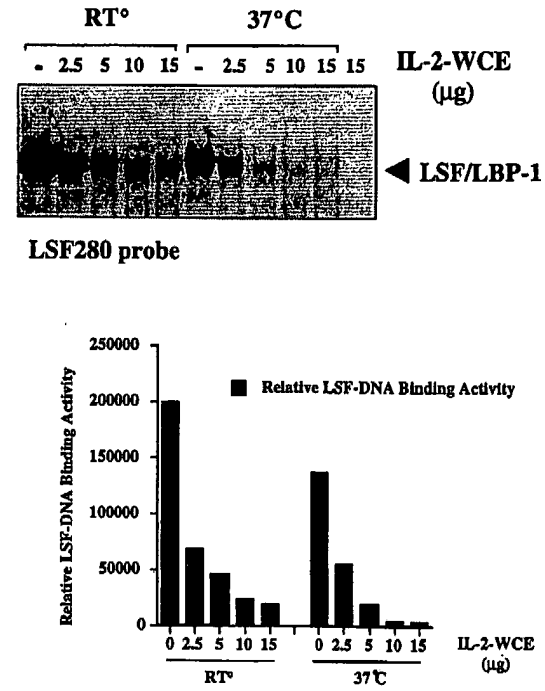

FIGURE 5. Inhibition of LSF/LBP-1 DNA binding activity of a normal healthy donor by the addition of IL-2-treated WCE from a seropositive patient to the binding reaction. *A*. EMSA using 5 μg of WCE from a normal healthy donor either alone (−) or in combination with increasing amount of WCE from donor 1 on day 5 of cycle 6. In the *last lane*, 15 μg of WCE from donor 1 on day 5 of cycle 6 was incubated at room temperature with the probe. *B*, Relative estimates of the LBP-1 (LSF)-DNA binding activity. Quantitation of the relative DNA binding activity of EMSA in *A* was performed by PhosphorImager (Molecular Dynamics) analysis.

patient 1 was performed using an anti-YY1 rabbit polyclonal Ab. The single sharp band corresponding to YY1 and visible on day 0 was no longer detectable on days 3 and 5 of the first cycle of IL-2 administration and on days 1, 3, and 5 of cycle 6, respectively (Fig. 4, *upper panel*). Several faint bands smaller than the expected 68 kDa were recognized by the anti-YY1 Ab, further supporting proteolytic cleavage of the transcription factor. After stripping the anti-YY1 Ab, the membrane was reprobed with an anti-actin polyclonal rabbit Ab. At all time points, a single band was clearly detectable (Fig. 4, *lower panel*), demonstrating that the IL-2-induced degradation was relatively specific for YY1. In addition, Western blot analyses were performed using WCE obtained from PBMC of HIV-seronegative healthy donors that were either left unstimulated or were stimulated in vitro for 1, 3, and 5 days with IL-2 (20 U/ml). The expression of YY1 protein during this in vitro culture period remained unaltered (data not shown).

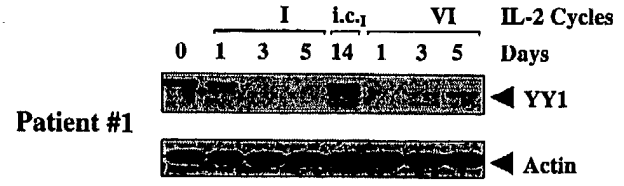

FIGURE 4. YY1 expression in an IL-2 treated HIV$^+$ patient. Immunoblot of WCE (10 μg) of PBMC isolated from donor 1 using anti-YY1 Abs (*upper panel*) and after stripping of the filter using anti-actin Abs (*lower panel*).

IL-2 induces either proteolysis or a potential inhibitor of LBP-1 (LSF)-DNA binding

We finally evaluated whether LBP-1 (LSF) was proteolytically cleaved by IL-2 administration by performing WCE-mixing EMSA. A concentration-dependent disappearance of the upper canonical band was indeed observed as a function of increasing the concentration of WCE from the HIV-infected individual. However, in contrast to what was observed for YY1, the disappearance of LBP-1 (LSF)/DNA binding was clearly concentration dependent at both room temperature and 37°C, although the effect was much stronger at 37°C than at room temperature (Fig. 5*A*). Quantitative analysis indeed demonstrated an almost complete lack of LBP-1 (LSF)-DNA binding at the two highest concentrations of IL-2-treated patients' WCE at 37°C (Fig. 5*B*). Unfortunately, we could not assess LBP-1 (LSF) expression, because the amount of WCE obtained from the HIV-infected patients required for the

detection of the LBP-1 isoforms was not sufficient to produce a detectable signal in Western blot experiments (data not shown).

Discussion

In the present study we have demonstrated that in vivo IL-2 administration induces a profound down-modulation of both YY1 and LBP-1 (LSF)-DNA binding activities to their specific cognate DNA elements. Evidence of a specific cleavage of YY1 was obtained by EMSA analysis after mixing different amount of WCE from IL-2-treated individuals and from normal seronegative donors and by Western blot analysis. In addition, a concentration-dependent disappearance of LBP-1 (LSF) binding was demonstrated in EMSA in the same WCE mixing experiments. Because no evidence of either YY1 or LBP-1 (LSF) down-modulation was observed after in vitro stimulation of PBMC from either HIV^- or HIV^+ individuals with IL-2, these effects are probably the result of a complex action of this cytokine when administered in vivo. No clear-cut correlation was noted between the levels of expression of the two transcription factors and the relative changes among PBMC subpopulations under IL-2 influence (Table I). Thus, the down-modulation of YY1 and LBP-1 (LSF) seems more likely caused by a soluble factor(s) induced by IL-2 administration or by a different activation state of the PBMC rather than by a different redistribution of cell subsets.

Evidence that WCE of IL-2-treated individuals contain a proteolytic activity that is responsible for YY1 degradation are here shown based on the results of the EMSA mixing experiments, in which the DNA binding of normal WCE was lost when IL-2-treated WCE was added to the reaction before the addition of the probe. By Western blot and EMSA analyses, we demonstrated that YY1 cleavage was relatively specific, because actin was intact and STAT5 binding activity unaltered, respectively, at all time points of IL-2 administration tested. In support of this hypothesis, a higher reduction of full-length YY1 binding to the DNA was observed when the extracts were incubated at 37°C. Moreover, quantitative analysis of the EMSA bands revealed that in contrast to the full-length YY1-UCR, the appearance of the truncated YY1Δ-UCR band was dependent on the amounts of IL-2 extracts added at both room temperature and 37°C, very likely as a consequence of the contribution of the truncated YY1 present in the IL-2-treated WCE. Conversely, quantitative analysis of the LBP-1 (LSF)-DNA binding activity demonstrated that the disappearance of the LBP-1 (LSF)-DNA binding was concentration dependent at both temperatures, although the effect was more evident at 37°C. One possible explanation is that the proteolytic activity required for YY1 degradation is not identical with that responsible for LBP-1 (LSF) cleavage and is still fully active at room temperature. Alternatively, the concentration-dependent decrease in LBP-1 (LSF)-DNA binding activity at room temperature might also depend upon a specific inhibitor of LBP-1 (LSF)-DNA binding induced in WCE of HIV-infected individuals receiving IL-2. In this regard, LBP-1d, lacking the DNA binding domain, was, however, shown to be capable of squelching the binding of the other LBP-1 isoforms by protein-protein interaction (12). Thus, we cannot rule out the possibility that in vivo IL-2 administration may result in a superinduction of either the LBP-1d isoform or analogous inhibitors.

The nature of the proteolytic activity present in IL-2-treated WCE is unknown at present, but it is very unlikely attributable to the HIV-associated protease for several reasons. First, the IL-2-treated patients were all receiving two RTI and one PI. Although beyond the scope of the present study, this antiviral regimen resulted in a sustained decrease in viremia (from 947 to <400 copies/ml at baseline and after 2 mo of therapy in patient 1, respectively; our manuscript in preparation). Furthermore, no increases in plasma viremia were observed during IL-2 administration. Second, the incubation of WCE from a normal healthy donor with WCE obtained from an HIV^+ individual receiving IL-2 but in whom YY1 was not degraded produced no reduction in either YY1- or LBP-1 (LSF)-DNA binding activities, suggesting that the effect was strictly dependent on IL-2 administration. Finally, only a minority of cells (1:100–10,000) are known to be infected in individuals at any particular time point (33, 34), and it is extremely unlikely that the viral protease present in these cells may override the pattern of protein expression of uninfected cells.

Among many genes transcriptionally regulated by YY1, the α-actin genes have been shown to be repressed by this transcription factor in analogy with the effect on the HIV-LTR (35). Of interest, proteolytic degradation of YY1 has been correlated with myogenic differentiation (35). In these cells, cleavage of YY1 during the myoblast-myotube transition allowed the expression of the sarcomeric α-actin genes, thus facilitating muscle development (35). In the same system the Ca^{2+}-dependent cysteine protease m-calpain has been clearly demonstrated as the enzyme responsible for the post-translational mechanism of YY1 down-regulation (35). Of note, the levels of this endopeptidase were also found increased after activation of T lymphocytes (36). Furthermore, in vitro activation-induced programmed cell death in PBMC from HIV^+ individuals has been shown to be specifically inhibited by calpain inhibitor II, suggesting that a calpain-dependent apoptosis might contribute to HIV-associated immunodeficiency (37). Although we did not observe YY1 degradation after IL-2 stimulation of patients' PBMC in vitro, we speculate that activation of circulating T cells of HIV^+-infected individuals by in vivo administration of IL-2 may increase the concentration of m-calpain, resulting in cleavage of YY1 and/or LBP-1 (LSF).

In conclusion, our findings are of potential interest for understanding the biochemical and immunological correlates of IL-2 administration to HIV-infected individuals. In this regard, it has been previously emphasized that one potential drawback of IL-2 administration observed in HIV-infected individuals was the transient increase in plasma HIV RNA levels frequently observed at the end of each cycle of i.v. infusion of IL-2 in the absence of ART (24). Our findings may provide a biochemical correlate of this phenomenon, i.e., the decreased expression or activity of two HIV transcriptional repressors such as YY1 and LBP-1 (LSF). Of note is the fact that increases in plasma viremia were not observed in any of our patients who received IL-2 by either s.c. or continuous infusion routes in the presence of ART (our manuscript in preparation). In addition, the copy numbers of both spliced and unspliced RNA, measured by competitive RT-PCR (38), remained unchanged in patients 2 (with 450 and 500 copies of unspliced RNA and 526 and 500 copies of fully spliced RNA/μg of total RNA after IL-2 vs baseline, respectively; data not shown), but were below the threshold of detection in patient 1 (40 copies of RNA/μg of total RNA) both before and after IL-2 treatment (data not shown), leaving open the question of whether IL-2 and IL-2-induced modulation of YY1 and LBP-1 (LSF) can significantly regulate HIV transcription in vivo in the presence of potent antivirals. Despite the fact the IL-2 is being considered as one of the most promising therapeutic agents to be associated with antiviral mixtures, little is known of its biological effects on HIV-infected individuals. Here, by showing a potential correlation between in vivo effects of IL-2 and down-modulation of YY1 and LBP-1 (LSF), we provide evidence that molecular markers can be found and should be further investigated to define novel sensitive correlates of the response to immunotherapy.

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fish[1], although many coloured areas used by stomatopods in communication contain ultraviolet components[14]. Most of the colour information from natural objects can be decoded by just four types of photo-receptor in the 300–700 nm range[12,15], so there must be other reasons for the bizarre retinal design of stomatopods, which probably uses 12 channels for colour. There are two possible explanations that logically extend the sensitivity range of 400 to 700 nm.

The first possibility is that stomatopod eyes examine colour space from 300 to 700 nm in much the same way as the ear examines auditory space. The multiple, narrow-band spectral sampling channels, from 300 to 700 nm, may be analogous to the different auditory frequencies to which a cochlea is tuned along its length. This may be thought of as a kind of 'digital' colour vision.

Alternatively, stomatopods may divide the spectral world from 300 to 700 nm into six dichromatically examined windows (two in the ultraviolet, mediated by row 1–4 R8 cells), each of which is subject to very fine spectral discrimination[14].

One of the ultraviolet sensitivities is in row 5 of the midband, a region of the eye that is probably used in polarization vision[4,5]. Anatomical[4] and microspectrophotometry[6] comparisons show that the same sensitivity exists in row 6 and that this is theoretically optimal for polarized-light vision[16].

As ultraviolet-blind humans, we have created a barrier in the spectrum at 400 nm. For the colour or polarization vision of stomatopods, this barrier is meaningless.

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AIDS

Re-emergence of HIV after stopping therapy

A dormant reservoir of human immunodeficiency virus (HIV) is established early on during primary infection[1] which consists of latently infected, resting CD4$^+$ T cells carrying replication-competent HIV. This pool can persist even in individuals who are receiving highly active antiretroviral therapy (HAART)[2–4]. Here we show that this pool rapidly re-emerges within weeks of discontinuing HAART in two patients, and that this re-emergence is associated with the appearance of HIV in the plasma (viraemia) of these patients. Both had been aviraemic while receiving HAART and intermittent treatment with interleukin-2 (ref. 5), and repeated attempts to isolate replication-competent HIV in this population of cells during therapy had been unsuccessful. This finding raises the possibility that there may be other tissue reservoirs of HIV that contribute to early plasma viral rebound following discontinuation of HAART in infected patients.

Despite the success of HAART in driving plasma viraemia to below the levels of detectability in many HIV-infected individuals[6,7], the persistence of a latent reservoir of HIV in resting CD4$^+$ T cells in HAART-treated individuals is considered to be a major impediment to the long-term control of HIV infection[8]. We have recently shown that intermittently administering interleukin-2 during continuous HAART reduces the size of this reservoir in resting CD4$^+$ T cells[5]. The only way to demonstrate that cellular reservoirs of HIV have been eradicated is by clinical trial in which therapy is discontinued in individuals who have become aviraemic with therapy, so we enrolled two patients from a previously reported cohort[5] in such a trial. This trial, involving 18 patients, will be the subject of a separate report[9].

Before HAART was discontinued in these two patients, we were unable to detect replication-competent HIV in the resting CD4$^+$ T cells of peripheral blood at two consecutive time points, despite culturing up to 330 million cells[5], and the virus could not be isolated from the lymph nodes of either patient[5]. After HAART was discontinued, we were able to detect plasma viraemia within three weeks in both patients.

We carried out quantitative co-culture assays[5,10] by using highly purified resting CD4$^+$ T cells at several time points. The pool of resting CD4$^+$ T cells carrying replication-competent HIV emerged shortly after plasma viraemia was detected (Fig. 1). This pool of cells increased in size by 3.8 log by week 4 and by 2.0 log by week 6 in patients 1 and 13 (numbered according to ref. 5), respectively, following the discontinuation of HAART. The integrated form of HIV DNA, which was not detected by Alu-LTR polymerase chain reaction (Table 1) in 10^6 resting CD4$^+$ T cells from either patient during HAART, was readily detectable during the reappearance of the virus in both patients (142 copies per million cells for patient 1 at week 6, and 420 copies for patient 13 at week 8).

Our results demonstrate the speed with which the latent HIV reservoir in the resting CD4$^+$ T-cell compartment emerges during the reappearance of plasma viraemia following discontinuation of HAART. This is as fast as the initial establishment of the reservoir in patients during primary infection[1]. Furthermore, given that replication-competent HIV was not detectable in resting CD4$^+$

Table 1 Quantitative analysis of integrated HIV-1 DNA in resting CD4$^+$ T cells

Patient	Integrated HIV-1 proviral load (copies per 10^6 cells)	
	Week 0	Week 6
1	<0.5	142
	Week 0	Week 8
13	<0.5	420

Genomic DNA from resting CD4$^+$ T cells was serially diluted and subjected to the polymerase chain reaction (PCR) in duplicate using nested 5′ primer from conserved Alu and 3′ primer from conserved HIV-1 long-terminal repeat (LTR) sequences as described[2]. A portion of the diluted first PCR product was further subjected to the second round of PCR using nested HIV-1 LTR-specific primers. The product of two rounds of PCR was detected by dot blotting using LTR-specific probe. Week 0 represents the time when patients discontinued HAART.

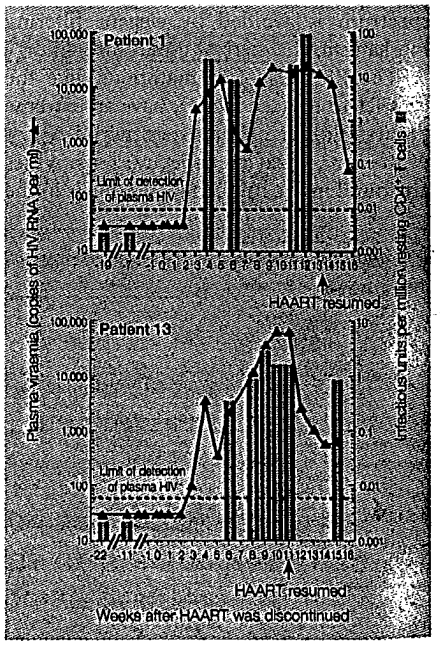

Figure 1 Rebound in plasma viraemia and re-emergence of the pool of latently infected, resting CD4$^+$ T cells in two patients after HAART was discontinued. Plasma HIV RNA was measured by using the bDNA assay (Chiron) with a detection limit of 50 copies per ml. Frequencies of resting CD4$^+$ T cells carrying replication-competent HIV were determined by quantitative co-culture assays as described[5,10]. Patients 1 and 13 received HAART for 33 and 30 months, respectively, before it was discontinued. Week 0 represents the time when patients discontinued HAART.

T cells from either the lymph nodes or peripheral blood before HAART was discontinued in these patients, it is possible that other tissue reservoirs are responsible for the rebound in plasma viraemia. Genotypic and phenotypic studies will be required to determine the source of this rebounding virus.

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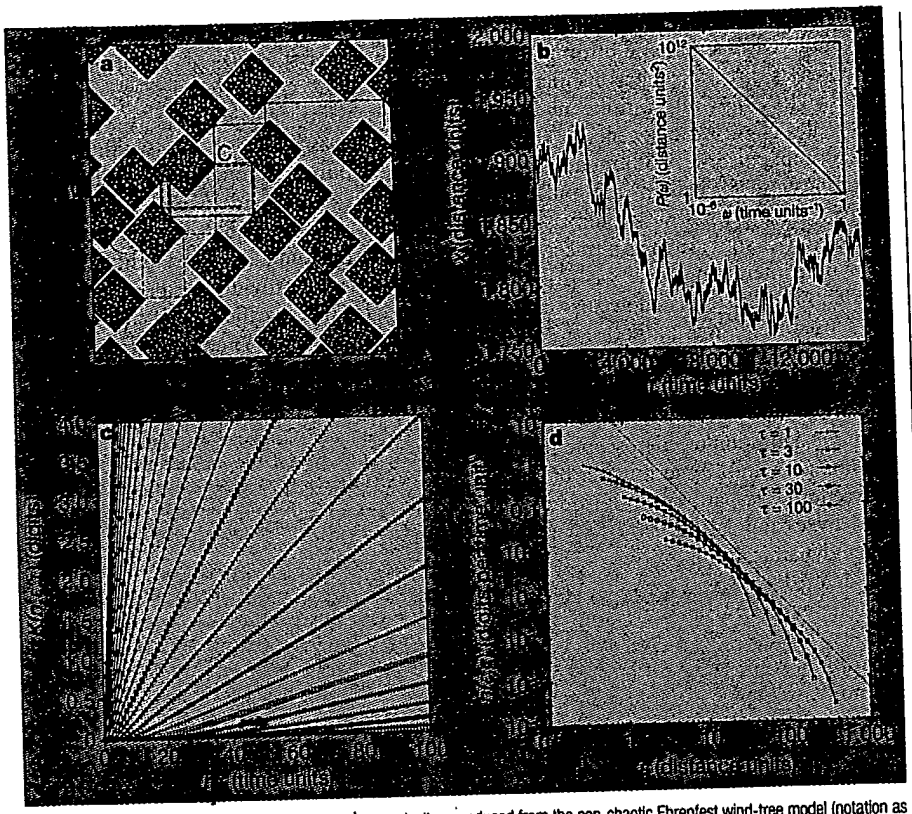

Figure 1 Brownian motion results of Gaspard *et al.*[1] numerically reproduced from the non-chaotic Ehrenfest wind-tree model (notation as in ref. 1). The square scatterers have a diagonal of two length units and fill half the area considered. The particle moves with unit velocity in four possible directions. The position on its trajectory is determined for 10^6 points separated by one time unit. **a**, Two nearby trajectories split only at a corner C; no exponential separation occurs (see Fig. 1 of ref. 1). **b**, A typical trajectory is diffusive with an ω^{-2} power spectrum (inset), where ω is the angular frequency (see Fig. 2 of ref. 1). **c**, The information entropy $K(n, \epsilon, \tau)$ for $\tau = 1$ and $\epsilon = 0.316 \times 1.21^m$, where m is an integer running from 0 to 25 (see Fig. 3 of ref. 1). **d**, The envelope of the slopes of these K curves, $h(\epsilon, \tau)$ appears to imply a positive (chaotic) h_{KS} for the Ehrenfest model, as for brownian motion (see Fig. 4 of ref. 1).

Statistical mechanics

Microscopic chaos from brownian motion?

Gaspard *et al.*[1] have analysed a time series of the positions of a brownian particle in a liquid, and claimed that it provides empirical evidence for microscopic chaos on a molecular scale. An accompanying comment[2] emphasized the fundamental nature of the experiment. Here we show that virtually identical results can be obtained by analysing a corresponding numerical time series of a particle in a manifestly microscopically non-chaotic system.

Like Gaspard *et al.*[1], we have analysed the position of a single particle colliding with many others. We used the Ehrenfest wind-tree model[3], in which the point-like ('wind') particle moves in a plane, colliding with randomly placed, fixed square scatterers ('trees') (Fig. 1a). We chose this model because collisions with the flat sides of the squares do not lead to exponential separation of corresponding points on initially nearby trajectories, so there are no positive Lyapunov exponents, which are characteristic of microscopic chaos. In contrast, Gaspard *et al.*[1] used a Lorentz model as being similar to brownian motion, a model in which the squares are replaced by hard, circular discs. This does exhibit exponential separation of nearby trajectories, leading to a positive Lyapunov exponent and hence microscopic chaos.

Nevertheless, despite being non-chaotic, the Ehrenfest model reproduces all the results presented by Gaspard *et al.*[1]. The particle trajectory segment shown in Fig. 1b is strikingly similar to that for the brownian particle (Fig. 2 of Gaspard *et al.*). Our subsequent analysis parallels that of Gaspard *et al.*[1], where further details may be found.

The microscopic 'chaoticity' is determined by estimating the Kolmogorov–Sinai entropy h_{KS} as described[4,5] by using the information entropy $K(n, \epsilon, \tau)$ obtained from the frequency with which the particle retraces part of its (previous) trajectory within a distance ϵ, for n measurements spaced at a time interval τ. As h_{KS} here equals the sum of the positive Lyapunov exponents, the determination of a positive h_{KS} would imply microscopic chaos. Like Gaspard *et al.*[1], we find that K grows linearly with time (Fig. 1c), giving a positive (non-zero) bound on h_{KS} (Fig. 1d). Indeed, Fig. 1b–d for a microscopically non-chaotic model are virtually identical to the corresponding Figs 2–4 of Gaspard *et al.* Thus, Gaspard *et al.* did not prove the presence of microscopic chaos in brownian motion.

The algorithm of refs 4,5 as applied here cannot determine the microscopic chaoticity of brownian motion because the time interval between measurements, 1/60 s (ref. 1), is so much larger than the microscopic timescale determined by the inverse collision frequency in a liquid, which is approximately 10^{-12} s. A decisive determination of microscopic chaos would require a time interval τ of the same order as characteristic microscopic timescales.

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Gaspard *et al.*[1] have shown that the position of a brownian particle behaves like a Wiener process with positive resolution-dependent entropy[2]. More surprisingly[3–5], they claim that this observation provides proof of 'microscopic chaos', a term they illustrate by examples of finite dimensional dynamical systems which are intrinsically unstable. We do not believe that they have provided evidence for microscopic chaos in the sense in which they use the term.

Although the recent literature finds such